MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4589–4596 Vol. 17, No. 8
0270-7306/97/$04.00+0

CCAAT-Binding Factor NF-Y and RFX Are Required for In Vivo Assembly of a Nucleoprotein Complex That Spans 250 Base Pairs: the Invariant Chain Promoter as a Model

MICHAEL W. LINHOFF, KENNETH L. WRIGHT, AND JENNY P.-Y. TING*

Lineberger Comprehensive Cancer Center, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Received 17 December 1996/Returned for modification 23 January 1997/Accepted 7 May 1997

The events that lead to promoter accessibility within chromatin are not completely understood. The invariant chain (Ii) promoter was used as a model to determine the contribution of different DNA-binding factors in establishing occupancy of a complex promoter. Gamma interferon induction of the Ii promoter requires the cooperation of multiple *cis* elements including distal S, X, and Y/CCAAT elements along with proximal GC and Y/CCAAT elements. The heteromeric transcription factor NF-Y binds to both Y/CCAAT elements. Genomic footprinting was used to analyze in vivo protein-DNA contacts for integrated Ii promoters bearing mutations in each element. The results reveal a hierarchy of transcription factor loading with NF-Y binding to the distal Y/CCAAT element being required for establishing protein-DNA interactions over the entire 250 bp analyzed. Mutation of the X box disrupts binding primarily at the adjacent Y/CCAAT element along with a lesser effect on GC box binding. Importantly, this finding is verified with a cell line which lacks a functional X-box-binding factor, RFX, providing physiological validity for the strategy described here. Mutation of both the S element and the GC box results in either no or little effect on transcription factor binding. However, mutation of the proximal Y/CCAAT element disrupts binding to the adjacent GC box and partially reduces binding in the distal S/X/Y domain. The crucial role for NF-Y in establishing promoter occupancy may be related to its histone fold motif, the essential component for assembling nucleosome-like structures.

In order to recruit RNA polymerase II to the appropriate site for initiation of transcription, multiple proteins of diverse structures must first recognize their cognate DNA sequences in *cis*-acting regulatory regions. In eukaryotic nuclei, these preliminary events are complicated by the overall complexity of the genome, which offers the potential for titration via nonspecific and specific interactions, and packaging of regulatory sequences into chromatin, which can influence the accessibility of binding sites. It has been proposed that the solution to these complications involves the assembly of highly organized nucleoprotein complexes which result from a combination of protein-protein and protein-DNA interactions facilitated by the arrangement of *cis*-acting elements within a promoter (21, 27, 54). Analyses of several enhancers have shown that some DNA-binding proteins function principally as structural components in organizing these short stretches of DNA into functional nucleoprotein complexes. Virus induction of beta interferon (IFN-β) transcription requires the assembly of an enhanceosome (52). The IFN-β enhancer requires a precise stereospecific alignment of regulatory sequences and the cooperation of at least three different transcription factors and the high-mobility group protein HMG I(Y) (18). HMG I(Y) promotes the cooperative assembly of factors on the promoter by binding to multiple sites in the enhancer and reversing intrinsic DNA bends (23). In a separate system, assembly of the T-cell receptor α enhancer requires the architectural protein lymphoid enhancer-binding factor 1 (LEF-1) (25). The HMG domain of LEF-1 induces a 130-degree bend upon DNA binding (24, 34), providing a mechanism for arranging the apposition of distant regulatory elements. Spatial organization of distant elements also can be influenced by the organization of regulatory sequences into nucleosomes. Transcription of the *Xenopus* vitellogenin gene in vitro requires a positioned nucleosome that loops out the DNA between enhancer elements and the core promoter which reside in linker regions (46).

Transcriptional regulation of genes involved in the major histocompatibility complex (MHC) class II antigen presentation pathway offers an intriguing model for analyzing promoter assembly (5, 26, 35, 53). Efficient presentation of exogenous antigen to T helper cells requires the coordinate expression of multiple genes including the α/β chains of DR, DQ, and DP, the MHC class II-associated invariant chain (Ii), and the α/β chains of DM. These genes are developmentally regulated with constitutive expression in mature B cells and inducible regulation in a variety of cell types. Three promoter elements within each of these genes, S (W), X, and Y, are critical for B-cell-specific and IFN-γ-induced expression. The sequence and spacing between these elements are highly conserved, and functional studies have indicated that these elements act synergistically. Insertions and deletions that alter the spacing between X and Y have shown that helical alignment between these two elements is required for function, and any spacing change between S and X is not tolerated (56, 57). Biochemical results suggest that physical interaction between the factors binding at X and Y does occur and that binding at the X and Y elements is cooperative (45, 60).

The Y element is a CCAAT box which is bound by the heteromeric DNA-binding protein NF-Y (also known as CBF, CP1, and YEBP) (13, 36, 63). Unlike the transcription factors C/EBP and CTF/NF1, which also bind CCAAT-like sequences, NF-Y exhibits a strict sequence requirement for this pentanucleotide (16). Binding sites for this factor have been described for several eukaryotic genes, and a statistical analysis

* Corresponding author. Mailing address: Lineberger Comprehensive Cancer Center, Department of Microbiology and Immunology, CB 7295, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. Phone: (919) 966-3036. Fax: (919) 966-3015.

of 502 unrelated eukaryotic promoters determined that Y/CCAAT sequences frequently are observed in the promoter-proximal sequences of these genes (9). NF-Y is composed of three subunits, NF-YA, -B, and -C, each of which is required for DNA binding (37, 49). Each of the subunits contains primary sequence that has remained highly conserved throughout evolution. In fact, subunits of the mammalian CCAAT-binding factor can substitute for the homologous subunits of the yeast CCAAT-binding factor, HAP2/3/5, in DNA-binding assays (14, 49). The conserved core sequences of NF-YC and NF-YB contain a 70-amino-acid region that displays similarity to the histone fold motif of the nucleosome subunits H2A and H2B, respectively (4, 48). The unique structure and evolutionary conservation of this transcription factor suggest that it plays a fundamental role in transcription of eukaryotic genomes.

The X box is composed of two distinct DNA-binding elements, X1 and X2. The X1 element is bound by the heteromeric transcription factor RFX (19). The absolute requirement for RFX in regulation of MHC class II genes is exemplified by the lack of X-box binding in cell lines derived from patients with bare lymphocyte syndrome (44). One subunit of this factor, RFX5, has recently been cloned by complementation of an immortalized patient line (50), and a second subunit, RFXAP, has been cloned by complementation of an in vitro-mutagenized B-lymphoblastoid cell line (20). The X2 element is located immediately 3′ of X1 and is similar in sequence to TRE/CRE sequences. A number of proteins have been described which bind to this sequence, although the recently purified X2BP cooperatively binds this site in the presence of purified RFX (39). Transcriptional regulation of MHC class II genes also requires the activity of the class II transactivator (CIITA) (51). This factor does not seem to bind DNA, and its mode of action remains unknown.

We previously examined the requirements of the S, X, and Y/CCAAT elements in assembling a relatively simple minimal DRA promoter in vivo (60). Cells harboring integrated mutant DRA promoters were used to observe changes in promoter occupancy which occur upon mutation of each element. Binding at the Y/CCAAT element was required for transcription factor occupancy at the X1 and X2 sites. Occupancy at the X1 and X2 elements is codependent, while mutation at either of these elements has a small effect on Y/CCAAT occupancy. This analysis led to a description of the NF-Y/CCAAT box interaction as a primary event in establishing occupancy of a simple promoter.

Our previous result established the interrelationship between X and Y/CCAAT elements in the DRA gene, which spans a mere 60 bp. However, many questions were left unanswered. For example, do all Y/CCAAT elements have this effect on the binding of other elements? How far is this effect observed? Is this effect dependent on adjacent sequences? Since only a limited region was analyzed, these issues were not addressed. These questions are now examined in the present report, which is made possible only because the invariant chain promoter is unique in that (i) two NF-Y (CBF) target sites are present, one at the conventional site at −50 to −80 bp and one further upstream at −200 bp; (ii) sequences adjacent to these two Y/CCAAT boxes are different, thus permitting us to examine the issue of how promoter context influences promoter loading; and (iii) the entire promoter region spanning 250 bp yields clear in vivo footprints, conferring a degree of resolution at the nucleotide level that is not attainable with other techniques. The results demonstrate a hierarchy of promoter loading with both inter- and intradomain dependencies. Importantly, NF-Y binding is shown to be the most critical step in promoter assembly.

MATERIALS AND METHODS

Plasmids. Site-specific mutation of the S, X, and distal Y/CCAAT elements has been described (6). Site-specific mutation of the GC box and proximal Y/CCAAT element were described previously (58). The Ii 395-CAT constructs were prepared by digestion of the Ii 790-CAT constructs with *Sac*I. This resulted in removal of a 400-bp fragment, which allowed for efficient primer extension into the invariant chain promoter using primers specific for the Bluescript KS vector.

Cell culture and the production of stable cell lines. U-373 MG is a glioblastoma multiform cell line which expresses high levels of MHC class II antigens upon IFN-γ induction. These cells were cultured by using McCoy's 5A medium supplemented with 8% fetal bovine serum and 2 mM glutamine. To generate cell lines with integrated promoters, 3×10^6 cells were transfected with 20 μg of each supercoiled Ii 395-CAT plasmid by electroporation (200 mV, 960 μF, 300 μl of complete medium; Bio-Rad Gene Pulser). To provide resistance for drug selection, 1 μg of the neomycin resistance vector pSV2neo was cotransfected with each construct. Two days posttransfection, selection was initiated by addition of 300 U of G418 (Geneticin; Gibco) per ml of medium. After 12 to 14 days, well-separated colonies were isolated by using cloning cylinders and amplified. Monoclonal lines were maintained in the presence of G418, and the drug was removed only prior to experimental assays. Genomic DNA was isolated from monoclonal lines, and clones were screened by PCR analysis using the Bluescript vector-specific primer KSVEC and the chloramphenicol acetyltransferase (CAT)-specific primer pCAT1.

The 2fTGH and G1B lines have been described (38). These cells were cultured in Dulbecco's modified Eagle medium supplemented with 8% fetal bovine serum and 2 mM glutamine.

In vivo genomic footprinting. Dimethyl sulfate (DMS) treatment of cells and genomic-DNA preparation were performed as described elsewhere (43). Cleaved genomic DNA was amplified by using the ligation-mediated PCR (LMPCR) protocol (40) with modifications (59). Cells (10^7) were treated for 24 h with 500 U of recombinant IFN-γ (Genentech) per ml before DMS treatment and genomic DNA isolation. The CAT-specific primer set used to visualize contacts for the upper strand of the proximal promoter is as follows: pCAT1, CAACGGTGGTATATCCAGTG (melting temperature [T_m] = 60°C); pCAT2, GCTTCCTTAGCTCCTGAAAATCTCGC (T_m = 63°C); pCAT3, CTTCCTTA GCTCCTGAAAATCTCGCCAAGC (T_m = 65°C). For protein-DNA contacts on the upper strand in the distal promoter domain, the CAT-specific primer pCAT1 was used along with the invariant-chain-specific primers IiUDP2, ACT TGGTAGATGTGGAAGTGAAAGC (T_m = 60°C), and IiUDP3, TTGGTAG ATGTGGAAGTGAAAGCTACAAAGGC (T_m = 64°C). Protected regions on the lower strand in the distal promoter domain were detected with the Bluescript-specific primer KSVEC, CTATAGGGCGAATTGGAG (T_m = 58°C) and the invariant-chain-specific primers IiLDP2, CAAAGTGCTTTCCTGTCTAG GGAGTGGAC (T_m = 66°C), and IiLDP3, GTGCTTTCCTGTCTAGGGAG TGGACATTTGC (T_m = 67°C). Footprinting of the endogenous invariant chain promoter for U373-MG, 2fTGH, and G1B was performed with previously described primer sets (7). Radiolabelled LMPCR products were separated by using 6% denaturing polyacrylamide gels.

CAT assays. Individual monoclonal cell lines were removed from selectable media prior to analysis. Cells were either untreated or cultured in the presence of 500 U of IFN-γ per ml. Forty hours after treatment, cells were harvested with trypsin and washed with phosphate-buffered saline. The cell pellets were resuspended in 100 mM Tris, pH 7.8, and lysed by freezing and thawing. Extracts were heat inactivated at 60°C for 10 min. Reaction mixtures were incubated at 37°C for 5 h. CAT activity was first analyzed by a xylene extraction-based scintillation technique (10, 41) using 75 μM unlabelled acetyl coenzyme A in conjunction with 25 μM [^{3}H]acetyl coenzyme A (NEN). Greater sensitivity was achieved by using [^{14}C]chloramphenicol, and these results are shown. Acetylated chloramphenicol was resolved by thin-layer chromatography and analyzed with a Molecular Dynamics PhosphorImager.

RESULTS

The invariant chain promoter shares the S/X/Y motif found in all MHC class II promoters; however, the 3′ boundary of these sequences is located 134 bp further upstream of the start site. At least six additional elements have been described within the intervening 200 bp of the invariant chain promoter. These include two NF-κB/Rel sites (7, 17, 42), a putative interferon consensus sequence binding protein site (3), a GC box and an imperfect Y/CCAAT element (58), and a putative TATA box. In this report, the imperfect Y/CCAAT element at −50 will be referred to as the proximal Y/CCAAT element while the Y/CCAAT element centered at position −200 as part of the S/X/Y motif will be referred to as the distal Y/CCAAT element. Full expression of invariant chain requires the GC box (−70) and the proximal Y/CCAAT element (−50). These

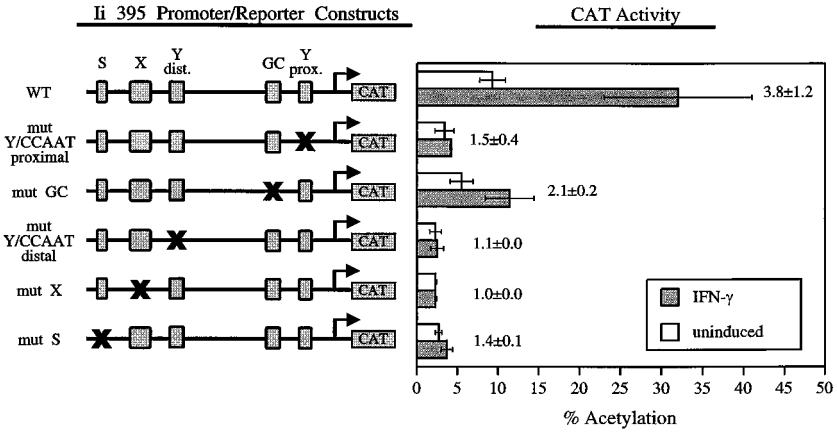

FIG. 1. Functional requirement of individual *cis*-acting elements for IFN-γ-induced transcription of stably integrated invariant chain promoter activity. A schematic of the Ii 395 promoter constructs used in this report is shown on the left. Site-specific disruption of individual elements is indicated (×). CAT activity is reported as the mean for three independent clones from each type of cell line (four for the wild type [WT] and the GC mutant [mut GC]) with standard error of the mean. The mean fold induction is provided to the right of each set of bars. dist., distal; prox., proximal.

sites function synergistically, and they interact with the transcription factors Sp1 and NF-Y, respectively (58).

Generation of stable transfectants for in vivo genomic footprinting analysis. In order to assess the roles of individual *cis*-acting elements in assembly of the IFN-γ-induced invariant chain promoter, monoclonal cell lines were generated that had stable integration of wild-type and mutant invariant chain promoters fused to a CAT reporter gene. Each of the Ii 395-CAT constructs (Fig. 1) was individually transfected into the U-373 MG cell line with a selectable marker, and clonal lines were isolated from each transfection. Ninety-five individual lines were created. Genomic DNA was isolated from each line and screened for plasmid integration by PCR analysis using vector- and CAT-specific primers flanking the promoter. Thirty-five lines exhibited productive integration. These lines were subsequently screened by DMS genomic footprinting, and seven lines were eliminated based on high-copy-number integration. High-copy-number lines were eliminated to prevent titration of transcription factors which may alter the integrity of genomic footprinting (55, 60). For each transfected construct, multiple clones were isolated to ensure that reproducible results would be obtained in the subsequent genomic footprinting analysis. The following numbers of clones were examined by in vivo footprinting: five clones harboring the wild-type construct, three with the mutated proximal Y/CCAAT construct, five with the mutated GC construct, two with the mutated distal Y/CCAAT construct, three with the mutated X construct, and three with the mutated S construct. All the in vivo footprints were performed at least twice. Although the level of reporter CAT expression varied significantly among clones, it is noteworthy that the in vivo footprints were remarkably similar when different clones harboring the same construct were analyzed. Thus, the site of integration did not appear to significantly alter the invariant chain footprint patterns.

In vivo functional activity of invariant chain promoter/reporter constructs. Transient transfection analysis of the invariant chain promoter previously determined that the distal S, X, and Y/CCAAT elements are required for IFN-γ induction in the U-373 MG cell line (8) and that additional Sp1 and NF-Y binding sites are crucial for promoter function in B-lymphoblastoid cell lines (58). In order to determine the relative functional contributions of each of these elements for IFN-γ induction of integrated invariant chain promoter activity, individual clonal lines were assayed for CAT activity with and without IFN-γ treatment (Fig. 1). Integrated wild-type promoters were inducible by IFN-γ, with the levels of induction for individual clonal lines being 6.9-, 2.9-, 1.3-, and 4.0-fold. As expected, mutation within each of the distal S, X, and Y/CCAAT elements results in disruption of IFN-γ inducibility and minimal promoter activity. Mutation of the proximal Y/CCAAT element also greatly reduces IFN-γ inducibility and promoter activity, while integrated promoters with a mutation in the GC box consistently retain partial inducibility, with values ranging from 1.8- to 2.5-fold.

Integrated wild-type and endogenous invariant chain promoters exhibit indistinguishable patterns of in vivo protein-DNA contacts. Prior to assessing the role of individual *cis*-acting elements in establishing promoter occupancy, it was necessary to determine if the integrated wild-type Ii 395-CAT construct displayed in vivo protein-DNA contacts similar to those of the endogenous invariant chain promoter. Primer sets were directed either to the genomic sequences flanking the endogenous invariant chain promoter or to the CAT and vector sequences flanking the integrated invariant chain promoter (Fig. 2A). The focus of this analysis was detection of protein-DNA contacts at 24 h post-treatment with IFN-γ. At this time, invariant chain transcription is maximal (7), and protein-DNA contacts are maximal over the entire promoter (8, 58). Figure 2B compares in vivo protein-DNA contacts for both the endogenous invariant chain promoter and the integrated wild-type promoter. The multiple guanine protections and enhancements in the GC box and the single protected guanine in the proximal Y/CCAAT element were indistinguishable for the two promoters (for sequence, see Fig. 3A). Similar results were obtained for the upper and lower strands in the distal promoter region (data not shown). The results of this analysis confirm the validity of using integrated invariant chain promoters to study promoter assembly and provide a system to test the effects of mutation on this process.

***cis* mutations that affect transcription factor occupancy of proximal promoter elements.** Previous results had demonstrated that the invariant chain proximal promoter Y/CCAAT element and GC box are synergistic in function. Synergism between these factors can be explained in part by cooperative binding of Sp1 and NF-Y to these sites in vitro. Sp1 binding adjacent to this imperfect Y/CCAAT element prolongs NF-Y's off-rate 4.5-fold, and NF-Y stabilizes Sp1 binding to a similar extent (58). To determine if this cooperativity is also found in

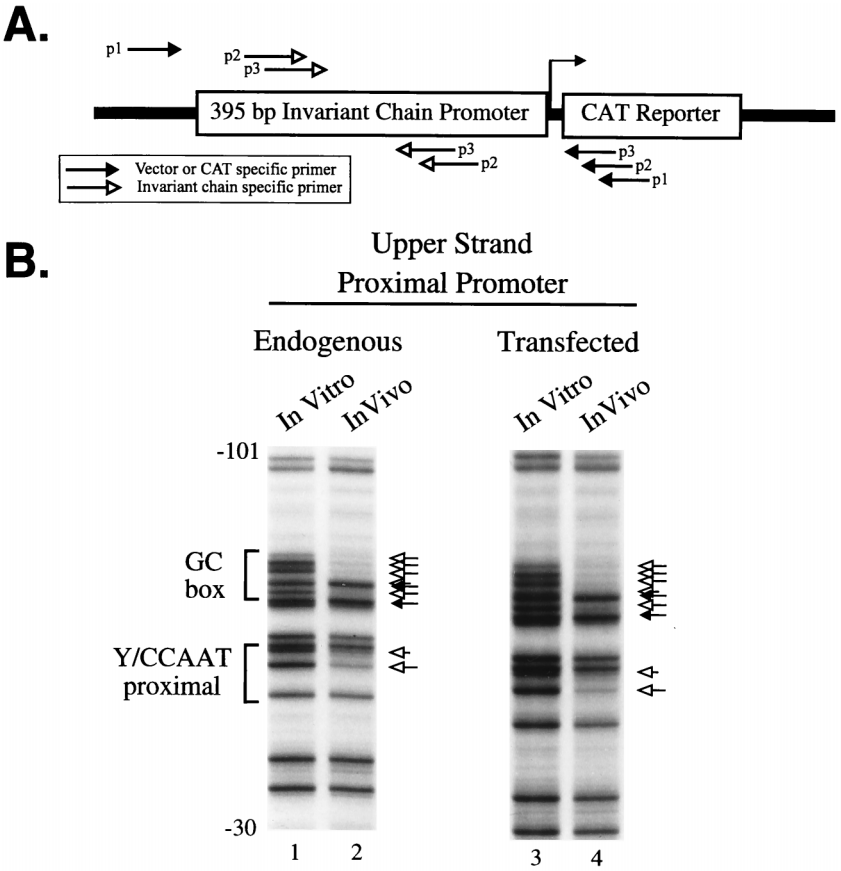

FIG. 2. In vivo footprinting of a stably integrated wild-type invariant chain promoter shows protein-DNA contacts identical to those of the endogenous promoter. (A) Schematic representation of an integrated invariant chain promoter. Arrows indicate the relative positions of primers used for in vivo footprinting. Filled arrows represent primers specific for the integrated plasmid vector and CAT sequences. Open arrows indicate primers specific for the human invariant chain promoter. p1, p2, and p3, primers 1, 2, and 3 of the LMPCR protocol. (B) In vivo genomic footprints of the upper strand in the proximal promoter domain. Lanes In Vitro, deproteinized genomic DNA methylated in vitro to reveal the complete guanine ladder. In vivo ladders were generated by using genomic DNA isolated from cells treated with DMS in culture. Lanes 1 and 2, amplification of the endogenous invariant chain promoter; lanes 3 and 4, amplification of the integrated wild-type invariant chain promoter. Open arrows, protected regions; filled arrows, enhancements. Partially protected regions or enhancements are indicated by shorter arrows. Results are reproducible for five wild-type cell lines.

vivo, stably integrated invariant chain promoters bearing mutations in either the proximal Y/CCAAT element or the GC box were analyzed by genomic footprinting. Protein-DNA contacts were analyzed only for the upper strand since the lower strand has relatively few protected regions in vivo (58). Mutation of the proximal Y/CCAAT element substantially disrupts Sp1 binding to the GC box, as the multiple strong guanine protections and enhancements are no longer detected (Fig. 3B, lanes 3 and 4). Mutation of the GC box results in a loss of protection within the GC box as expected, and the protection of the single guanine in the Y/CCAAT element was reduced but not eliminated (Fig. 3B, lanes 5 and 6). These results show that Sp1 binding in vivo is dependent on NF-Y binding at the adjacent Y/CCAAT element and that NF-Y binding is enhanced by Sp1 binding but is not dependent on it.

The distal Y/CCAAT element and X box were analyzed for their roles in establishing transcription factor occupancy in the proximal promoter domain. Although the distal Y/CCAAT element is 154 bp away from the proximal Y/CCAAT element, mutation of this site completely eliminates any detectable protection of the proximal Y/CCAAT element and GC box (Fig. 3B, lanes 7 and 8). When the X box is mutated, however, partially protected regions in both the proximal Y/CCAAT element and the GC box are consistently observed (Fig. 3B, lanes 9 and 10). These results show that DNA-binding events in the distal promoter domain are critical for transcription factor occupancy within the proximal promoter domain, with NF-Y binding to the distal Y/CCAAT element being absolutely required for this event.

***cis* mutations that affect transcription factor occupancy of distal promoter elements.** Transcription factor contacts within the proximal promoter domain were affected by all of the mutations tested above, albeit to different degrees. A similar analysis was undertaken to assess the role of individual *cis*-acting elements in establishing transcription factor occupancy of the distal promoter domain. It was of interest to determine to what extent could proximal promoter mutations influence in vivo binding within the distal promoter domain. Mutation of the proximal Y/CCAAT element results in partially reduced protein-DNA contacts in the distal Y/CCAAT element and X box. On the lower strand the two protected guanines in the distal Y/CCAAT element and three protected guanines in the X box are consistently and partially reduced but not eliminated (Fig. 4B, lanes 3 and 4). Although the distal Y/CCAAT element shows no protected regions on the upper strand, contacts are detected within the X box (Fig. 4C, lanes 3 and 4). In contrast, when the GC box is mutated, the lower-strand guanine protections within the distal Y/CCAAT element and X

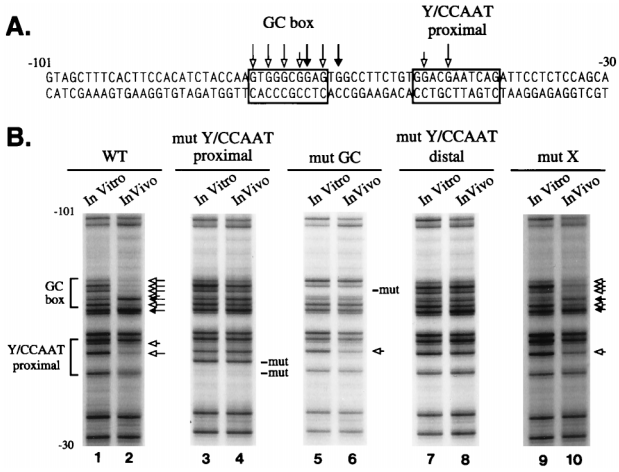

FIG. 3. In vivo genomic footprinting of stably integrated mutant invariant chain promoters reveals the requirement for NF-Y binding in establishing transcription factor occupancy in the proximal promoter domain. (A) Proximal promoter sequence and summary of in vivo protein-DNA interactions revealed by DMS footprinting. *cis*-acting elements are boxed. Open arrows, protected regions; filled arrows, enhancements. (B) In vivo footprints on the upper strand in the proximal promoter domain. The cell line is indicated above each pair of lanes. Contacts indicated for each type of integrated promoter were consistent for at least two independent cell lines. Visible sequence alterations resulting from mutations (mut) are indicated. WT, wild type. All other symbols are as described for Fig. 2B.

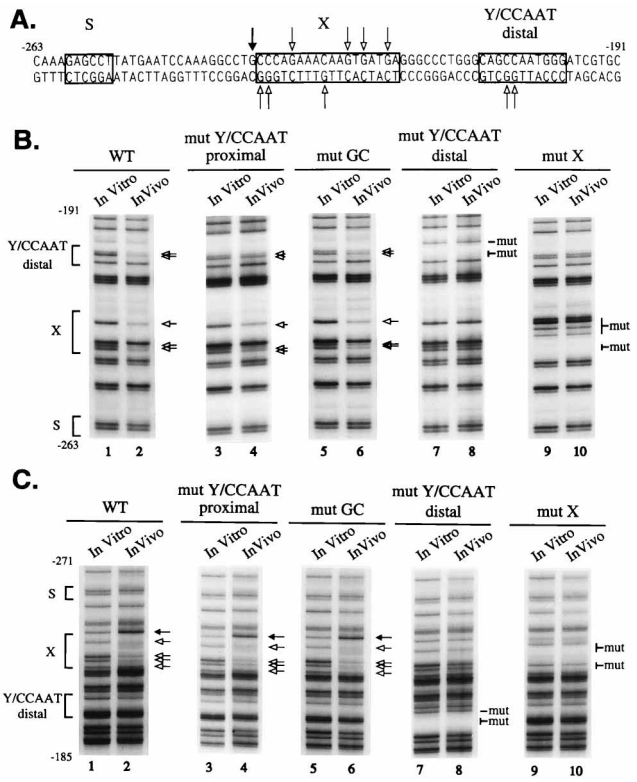

FIG. 4. In vivo genomic footprinting of stably integrated mutant invariant chain promoters reveals significant disruption of protein-DNA contacts in the distal promoter domain upon mutation of distal elements but not proximal elements. (A) Distal promoter sequence and summary of in vivo protein-DNA interactions revealed by DMS footprinting. The S, X, and Y/CCAAT elements are boxed. All other symbols are as in Fig. 3A. (B) In vivo genomic footprints for the lower strand. (C) In vivo genomic footprints on the upper strand.

box are not significantly reduced (Fig. 4B, lanes 5 and 6). Likewise, the guanine enhancement at the 5′ boundary of the X box and four protected guanines within the X box are unchanged (Fig. 4C, lanes 5 and 6). These results show that while the proximal Y/CCAAT element can influence transcription factor binding in the distal promoter domain, the GC box has little to no long-distance effect on promoter occupancy.

The distal Y/CCAAT element and X box were next analyzed to determine their role in establishing transcription factor occupancy of the distal promoter domain. Mutation of the distal Y/CCAAT element completely disrupted adjacent X box binding. The three strongly protected guanines on the lower strand of the X box are lost (Fig. 4B, lanes 7 and 8). This result was confirmed by analysis of the upper strand where the guanine enhancement at the 5′ boundary of the X box and four protected guanines within the X box are no longer detected (Fig. 4C, lanes 7 and 8). Also, mutation of the X box results in a loss of detectable guanine protection in the distal Y/CCAAT element (Fig. 4B, lanes 9 and 10). These results provide strong evidence that in vivo transcription factor loading at the distal Y/CCAAT element and at the X box is codependent.

Mutation of the S element does not affect transcription factor occupancy. Although the S element is required for IFN-γ induction, in vivo protein-DNA contacts are not detected within the S element for both DRA and invariant chain (8, 59). The role of the S element in assembly of the active invariant chain promoter was studied by in vivo footprinting of integrated invariant chain promoters with a mutated S element. Protein-DNA contacts in both the proximal promoter (Fig. 5, lanes 1 and 2) and the distal promoter (Fig. 5, lanes 3 and 4) are identical to those obtained for the wild-type promoter, indicating that an intact S element is not necessary for promoter occupancy even though this element is absolutely required for IFN-γ induction and promoter function.

Physiologic validation of the X-box mutation results obtained with the RFX-deficient cell line G1B. One approach to verify the validity of the experiments described above is to compare results obtained by using the transfected mutant constructs with mutant cell lines harboring defects in the protein factors which bind to these sites. The G1B cell line lacks IFN-γ induction of MHC class II genes as well as the invariant chain

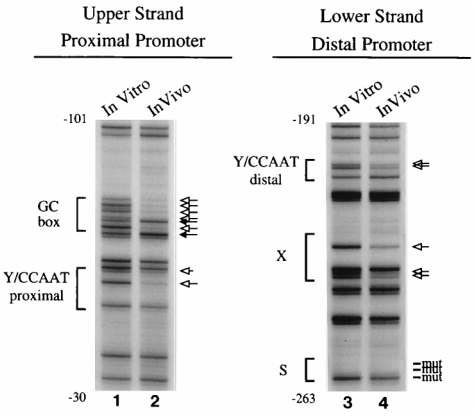

FIG. 5. In vivo genomic footprinting of stably integrated invariant chain promoters with site-specific mutation of the S element shows full transcription factor occupancy in both the proximal and the distal promoter domains. Footprints of the upper strand of the proximal promoter are shown in lanes 1 and 2. Footprints of the lower strand of the distal promoter are shown in lanes 3 and 4. Specific alterations in the S element can be visualized in the lower strand (mut). Individual contacts were reproducible for three independent cell lines.

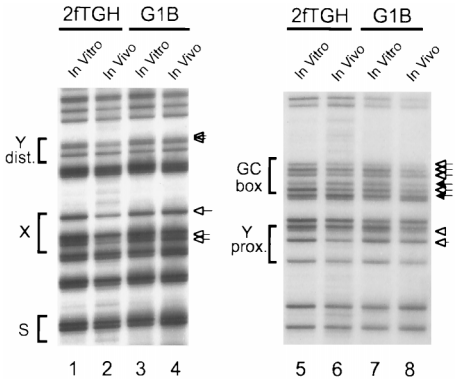

FIG. 6. The RFX-deficient cell line G1B displays a partially unloaded promoter upon IFN-γ induction. In vivo footprints are shown for both the distal (dist.) (lanes 1 to 4) and the proximal (prox.) (lanes 5 to 8) promoter regions of the endogenous invariant chain promoter. The parental cell line 2fTGH is included (lanes 1, 2, 5, and 6) to show contacts in a cell line where invariant chain transcription is induced normally. The results were repeated with two different DNA preparations for each cell line. The normal protected regions and enhancements are indicated (arrows).

(38). Nuclear extracts from G1B lack RFX binding activity (12), and more recent analysis has revealed that the RFX5 component of RFX is mutated in this cell line (5a). In vivo footprinting of the endogenous invariant chain promoter was performed by using the wild-type control, 2fTGH. In vivo footprints are detected over the X box and distal Y/CCAAT element (Fig. 6, lanes 1 and 2) and the GC box and proximal Y/CCAAT element (Fig. 6, lanes 5 and 6). The 2fTGH footprints are reproducibly weaker than those of the U-373 MG cell line used in all of the previous figures. A similar analysis using G1B shows a lack of footprints at both distal X and Y/CCAAT elements (Fig. 6 lanes 3 and 4); however, the proximal GC element is still partially occupied (Fig. 6, lanes 7 and 8). Thus, the invariant chain promoter is similarly disrupted both in cells defective in the X-box binding factor and in the transfected X-box mutation cell lines. This demonstrates that the integrated promoter analysis used in this report is physiologically valid.

DISCUSSION

In this study, we have analyzed the invariant chain promoter to determine the role of individual *cis*-acting elements in assembly of a complex nucleoprotein structure. This in vivo approach has defined a hierarchy of protein-DNA interactions over 250 bp of a promoter in which some mutations have localized effects on transcription factor binding, others have an impact over the entire promoter, and some have little to no effect. Our results show that the Y/CCAAT-binding factor NF-Y is absolutely required for transcription factor binding to adjacent sites as well as to sequences as far as 150 bp away.

Whereas functional analysis of the invariant chain promoter has shown that the distal S, X, and Y/CCAAT elements are equally critical for IFN-γ-induced expression, analysis of promoter occupancy by DMS footprinting has revealed that these elements do not equally contribute to establishing promoter occupancy. Mutation of the distal Y/CCAAT element in this motif entirely eliminates detectable protein-DNA contacts not only at the adjacent X box, but also at the Sp1 and NF-Y binding sites in the proximal promoter domain located 150 bp away. These results show that NF-Y binding to the distal Y/CCAAT element has a global role in factor association for the entire promoter. Mutation of the X box results in a significant disruption of protein-DNA contacts within the distal promoter domain, but only partial reduction of transcription factor binding in the proximal promoter domain is observed. This partial disruption could be explained by the fact that X-box mutation disrupts NF-Y binding to the distal Y/CCAAT element, thereby impairing the stabilizing effect that this element has on the proximal promoter domain. Finally, mutation of the S element resulted in no disruption of transcription factor binding to the invariant chain promoter. Despite the absence of an effect on promoter occupancy, IFN-γ induction and promoter activity are greatly reduced upon mutation of the S element. This indicates that an occupied promoter is not sufficient for transcriptional activation, and additional steps requiring a functional S element are necessary for promoter activity.

The codependency of promoter occupancy at the distal Y/CCAAT element and X box is in agreement with biochemical evidence which reveals physical interaction and cooperativity between the factors binding at X and Y (45, 60). It is noteworthy that others have observed that the invariant chain is expressed in B-lymphoblastoid cell lines which lack RFX (15, 29), although it is unclear to what level. In contrast, when an RFX-deficient, IFN-γ-responsive cell line was examined, invariant chain expression was abolished (38). Similarly, another set of mutants selected for the lack of IFN-γ-induced class II MHC expression are also defective for invariant chain induction (11). Differences between constitutive class II MHC expression and that induced by IFN-γ are observed with regard to the role of the master regulator, CIITA. It is widely accepted that CIITA does not influence promoter loading of the class II MHC genes. This is now found to apply only to B-cell lines, while CIITA is required for promoter loading in IFN-γ-inducible cell lines (57a). These experiments together lead us to conclude that there are fundamental differences between the mechanisms governing constitutive class II MHC and invariant chain gene expression and that induced by IFN-γ. One postulation is that constitutive expression of the invariant chain in B lymphocytes may serve an additional function in addition to antigen presentation. Indeed, recent evidence suggests that the invariant chain, but not MHC class II, is critical for late stages of B-cell development (47).

Another difference between our work with IFN-γ-inducible cells and the work of others with B-cell lines is that different results were obtained by using a similar strategy of footprinting transfected mutated promoters. We have consistently observed that the mutation of specific elements can influence the occupancy of other elements within the DRA promoter, and now with the invariant chain promoter. In vivo footprinting analysis of transfected DRA promoters in a B-lymphoblastoid line has shown that mutation of either the X or the Y/CCAAT element does not affect binding at other sites (31). This finding is unexpected because in bare lymphocyte syndrome cell lines, the lack of functional RFX is known to result in an unoccupied promoter (30, 32). The authors suggest that this may be due to differences between the integration locus and that of the MHC. In our studies using IFN-γ-inducible cells, mutation of X and Y/CCAAT elements has effects on the occupancy of other elements, and this is observed apparently regardless of integration site. Importantly, the result obtained with the transfected X-box mutation constructs is further confirmed with a mutant cell line lacking RFX. Thus, in the G1B cell line, the in vivo footprint of the invariant chain promoter is similar to that of the transfected promoter which bears a mutated X element.

Mutations of the proximal Y/CCAAT element and GC box reveal distinct effects on promoter occupancy. Mutation of the GC box only weakly disrupts binding to the proximal Y/CCAAT element, and little disruption of transcription factor

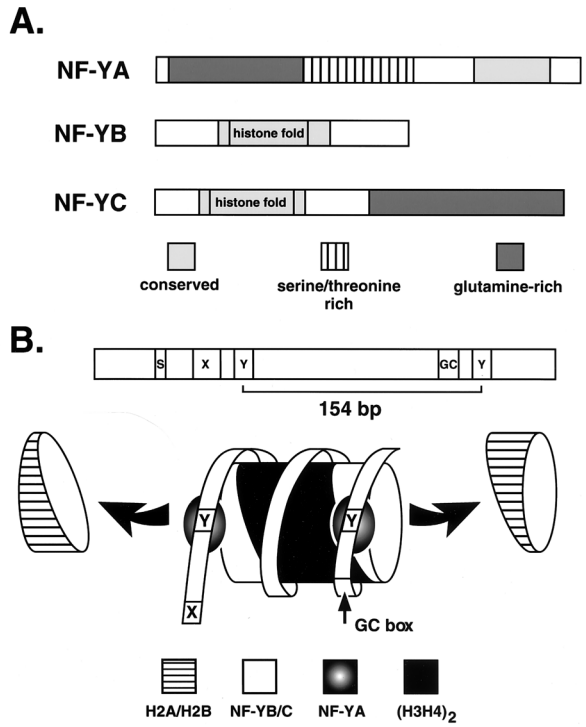

FIG. 7. Model for NF-Y function in regulating invariant chain promoter occupancy. (A) Graphic representation of the three protein subunits of NF-Y with indication of domains. "Conserved," evolutionarily conserved sequences in eukaryotic CCAAT-binding factors. The histone fold homology is found within the conserved portions of NF-YB/C. (B) Model for the role of NF-Y in establishing promoter occupancy in chromatin. The invariant chain promoter is shown as a ribbon with *cis*-acting elements boxed. The filled-in center represents the $(H3H4)_2$ tetramer which functions to organize 120 bp of nucleosomal DNA (28). The final 20 bp on either side in nucleosomes is usually organized by the H2A/H2B dimers. In this model, the H2A/H2B-like histone fold motifs of the NF-YB/C subunits interact with the nucleosome core, replacing the H2A/H2B subunits with sequence-specific binding by the NF-YA subunit positioning the nucleosome-like structure between the Y/CCAAT elements of the promoter, which are separated by 154 bp.

binding in the distal promoter domain is observed. These results are reflected in the partial IFN-γ inducibility that is retained by integrated GC mutation promoters. The proximal Y/CCAAT element, however, plays a significant role in establishing promoter occupancy, having both a local effect in that it is required for Sp1 binding to the neighboring GC box as well as a stabilizing effect for factors binding to the distal Y/CCAAT element and X box. The in vivo codependency of binding to the proximal Y/CCAAT element and GC box agrees with their in vitro cooperative binding shown previously (58).

Why does NF-Y binding play such a critical role in helping other transcription factors to bind, and how can it mediate this effect over 150 bp? The answer may lie in the unique structure of NF-Y. DNA binding is mediated by the evolutionarily conserved domains in each of the three subunits (Fig. 7A). The conserved sequences of NF-YB and NF-YC show sequence similarity with the histone fold motifs of the histone proteins H2B and H2A, respectively (4, 48). This structural motif has emerged as the fundamental architectural domain of the nucleosome (1, 2). A number of proteins share this motif, including several TFIID-associated factors (4). Indeed, dTAFII42 and dTAFII62 have been shown to adopt the histone fold and form tetrameric structures resembling the nucleosome core (61). Since the nucleosome octamer is assembled via the reversible association of H2A/H2B dimers with a central $(H3H4)_2$ core tetramer (22), the histone fold motifs of NF-YB and NF-YC may allow for interactions with the core tetramer (49). We suggest a speculative model for the invariant chain promoter (Fig. 7B) wherein sequence-specific binding mediated by the NF-YA subunit (62) could result in the formation of a Y/CCAAT element-positioned nucleosome-like structure allowing for efficient transcription complex formation. It is interesting to note that simultaneous mutation of two Y/CCAAT elements in the *Xenopus hsp70* promoter results in disruption of chromatin, yielding an altered pattern of micrococcal nuclease cleavage (33).

In conclusion, the analysis of mutant promoter constructs by in vivo genomic footprinting represents a powerful and high-resolution approach for dissecting the contributions of individual *cis*-acting elements in establishing promoter occupancy. This approach has revealed that Y/CCAAT element binding by NF-Y is a required event for assembly of a complex promoter. This requirement is influenced by the context of the promoter. The X element also has far-reaching effects on promoter loading although less than that of the Y element.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI29564. J.P.-Y.T. is the recipient of an American Cancer Society Faculty Award. K.L.W. is an Arthritis Foundation Postdoctoral Fellow. M.W.L. was supported by NIH predoctoral training grant 5-T32 GM08332.

M.W.L. thanks Debra Taxman for critically reviewing the manuscript and Cheryl Skinner for providing methylated 2fTGH and G1B DNAs.

REFERENCES

1. **Arents, G., R. W. Burlingame, B.-C. Wang, W. E. Love, and E. N. Moudrianakis.** 1991. The nucleosomal core histone octamer at 3.1 Å resolution: a tripartite protein assembly and a left-handed superhelix. Proc. Natl. Acad. Sci. USA **88:**10148–10152.
2. **Arents, G., and E. N. Moudrianakis.** 1995. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. Proc. Natl. Acad. Sci. USA **92:**11170–11174.
3. **Barr, C. L., and G. F. Saunders.** 1991. Interferon-γ-inducible regulation of the human invariant chain gene. J. Biol. Chem. **266:**3475–3481.
4. **Baxevanis, A. D., G. Arents, E. N. Moudrianakis, and D. Landsman.** 1995. A variety of DNA-binding and multimeric proteins contain the histone fold motif. Nucleic Acids Res. **23:**2685–2691.
5. **Benoist, C., and D. Mathis.** 1990. Regulation of major histocompatibility complex class-II genes: X, Y, and other letters of the alphabet. Annu. Rev. Immunol. **8:**681–715.

5a. **Brickey, J.** Unpublished data.

6. **Brown, A. M., C. L. Barr, and J. P.-Y. Ting.** 1991. Sequences homologous to class II MHC W, X, and Y elements mediate constitutive and IFN-γ-induced expression of human class II-associated invariant chain gene. J. Immunol. **146:**3183–3189.
7. **Brown, A. M., M. W. Linhoff, B. Stein, K. L. Wright, A. S. Baldwin, P. V. Basta, and J. P.-Y. Ting.** 1994. Function of NF-κB/Rel binding sites in the major histocompatibility complex class II invariant chain promoter is dependent on cell-specific binding of different NF-κB/Rel subunits. Mol. Cell. Biol. **14:**2926–2935.
8. **Brown, A. M., K. L. Wright, and J. P.-Y. Ting.** 1993. Human major histocompatibility complex class II-associated invariant chain gene promoter: functional analysis and *in vivo* protein/DNA interactions of constitutive and IFN-γ-induced expression. J. Biol. Chem. **268:**26328–26333.
9. **Bucher, P.** 1990. Weight matrix description of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J. Mol. Biol. **212:**563–578.
10. **Cassinotti, P., and M. Weitz.** 1994. Increasing the sensitivity of a common CAT assay. BioTechniques **17:**36–40.
11. **Chang, C.-H., and R. A. Flavell.** 1995. Class II transactivator regulates the expression of multiple genes involved in antigen presentation. J. Exp. Med. **181:**765–767.
12. **Chin, K.-C., C. Mao, C. Skinner, J. L. Riley, K. L. Wright, C. S. Moreno, G. R. Stark, J. M. Boss, and J. P.-Y. Ting.** 1994. Molecular analysis of G1B and G3A IFNG mutants reveals that defects in CIITA or RFX result in defective class II MHC and Ii gene induction. Immunity **1:**687–697.
13. **Chodosh, L. A., A. S. Baldwin, R. W. Carthew, and P. A. Sharp.** 1988. Human CCAAT-binding proteins have heterologous subunits. Cell **53:**11–24.

14. **Chodosh, L. A., J. Olesen, S. Hahn, A. S. Baldwin, L. Guarente, and P. A. Sharp.** 1988. A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable. Cell **53:**25–35.
15. **de Préval, C., M. R. Hadam, and B. Mach.** 1988. Regulation of genes for HLA class II antigens in cell lines from patients with severe combined immunodeficiency. N. Engl. J. Med. **318:**1295–1300.
16. **Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis.** 1987. A multiplicity of CCAAT box-binding proteins. Cell **50:**863–872.
17. **Doyle, C., P. J. Ford, P. D. Ponath, T. Spies, and J. L. Strominger.** 1990. Regulation of the class II-associated invariant chain gene in normal and mutant B lymphocytes. Proc. Natl. Acad. Sci. USA **87:**4590–4594.
18. **Du, W., D. Thanos, and T. Maniatis.** 1993. Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell **74:**887–898.
19. **Durand, B., M. Kobr, W. Reith, and B. Mach.** 1994. Functional complementation of major histocompatibility complex class II regulatory mutants by the purified X-box-binding protein RFX. Mol. Cell. Biol. **14:**6839–6847.
20. **Durand, B., P. Sperisen, P. Emery, E. Barras, M. Zufferey, B. Mach, and W. Reith.** 1997. RFXAP, a novel subunit of the RFX DNA binding complex is mutated in MHC class II deficiency. EMBO J. **16:**1045–1055.
21. **Echols, H.** 1986. Multiple DNA-protein interactions governing high-precision DNA transactions. Science **233:**1050–1056.
22. **Eickbush, T. H., and E. N. Moudrianakis.** 1978. The histone core complex: an octamer assembled by two sets of protein-protein interactions. Biochemistry **17:**4955–4964.
23. **Falvo, J. V., D. Thanos, and T. Maniatis.** 1995. Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). Cell **83:**1101–1111.
24. **Giese, K., J. Cox, and R. Grosschedl.** 1992. The HMG domain of lymphoid enhancer factor-1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell **69:**185–195.
25. **Giese, K., C. Kingsley, J. R. Kirshner, and R. Grosschedl.** 1995. Assembly and function of a TCRα enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes Dev. **9:**995–1008.
26. **Glimcher, L. H., and C. J. Kara.** 1992. Sequences and factors: a guide to MHC class-II transcription. Annu. Rev. Immunol. **10:**13–49.
27. **Grosschedl, R.** 1995. Higher-order nucleoprotein complexes in transcription: analogies with site-specific recombination. Curr. Opin. Cell Biol. **7:**362–370.
28. **Hayes, J. J., D. J. Clark, and A. P. Wolffe.** 1991. Histone contributions to the structure of DNA in the nucleosome. Proc. Natl. Acad. Sci. USA **88:**6829–6833.
29. **Hume, C. R., L. A. Shookster, N. Collins, R. O'Reilly, and J. S. Lee.** 1989. Bare lymphocyte syndrome: altered HLA class II expression in B cell lines derived from two patients. Hum. Immunol. **25:**1–11.
30. **Kara, C. J., and L. H. Glimcher.** 1991. In vivo footprinting of MHC class II genes: bare promoters in the bare lymphocyte syndrome. Science **252:**709–712.
31. **Kara, C. J., and L. H. Glimcher.** 1993. Promoter accessibility within the environment of the MHC is affected in class II-deficient combined immunodeficiency. EMBO J. **12:**187–193.
32. **Kara, C. J., and L. H. Glimcher.** 1993. Three in vivo promoter phenotypes in MHC class II deficient combined immunodeficiency. Immunogenetics **37:**227–230.
33. **Landsberger, N., and A. P. Wolffe.** 1995. Role of chromatin and Xenopus laevis heat shock transcription factor in regulation of transcription from the X. laevis hsp70 promoter in vivo. Mol. Cell. Biol. **15:**6013–6024.
34. **Love, J. J., X. Li, D. A. Case, K. Giese, R. Grosschedl, and P. E. Wright.** 1995. Structural basis for DNA bending by the architectural transcription factor LEF-1. Nature **376:**791–795.
35. **Mach, B., V. Steimle, E. Martinez-Soria, and W. Reith.** 1996. Regulation of MHC class II genes: lessons from a disease. Annu. Rev. Immunol. **14:**301–331.
36. **Maity, S. N., P. T. Golumbek, G. Karsenty, and B. de Crombrugghe.** 1988. Selective activation of transcription by a novel CCAAT binding factor. Science **241:**582–585.
37. **Maity, S. N., S. Sinha, E. C. Ruteshouser, and B. de Crombrugghe.** 1992. Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT binding factor. J. Biol. Chem. **267:**16574–16580.
38. **Mao, C., D. Davies, I. M. Kerr, and G. R. Stark.** 1993. Mutant human cells defective in induction of major histocompatibility complex class II genes by interferon γ. Proc. Natl. Acad. Sci. USA **90:**2880–2884.
39. **Moreno, C. S., P. Emery, J. E. West, B. Durand, W. Reith, B. Mach, and J. M. Boss.** 1995. Purified X2 binding protein (X2BP) cooperatively binds the class II MHC X box region in the presence of purified RFX, the X box factor deficient in the bare lymphocyte syndrome. J. Immunol. **155:**4313–4321.
40. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246:**780–786.
41. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
42. **Pessara, U., and N. Koch.** 1990. Tumor necrosis factor α regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-κB-like factor to a promoter element. Mol. Cell. Biol. **10:**4146–4154.
43. **Pfeifer, G. P., R. L. Tanguay, S. D. Steigerwald, and A. D. Riggs.** 1990. In vivo footprint and methylation analysis by PCR-aided genomic sequencing: comparison of active and inactive X chromosome DNA at the CpG island and promoter of human PGK-1. Genes Dev. **4:**1277–1287.
44. **Reith, W., S. Satola, C. Herrero Sanchez, I. Amaldi, B. Lisowska-Grospierre, C. Griscelli, M. R. Hadam, and B. Mach.** 1988. Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. Cell **53:**897–906.
45. **Reith, W., C.-A. Siegrist, B. Durand, E. Barras, and B. Mach.** 1994. Function of major histocompatibility complex class II promoters requires cooperative binding between factors RFX and NF-Y. Proc. Natl. Acad. Sci. USA **91:**554–558.
46. **Schild, C., F.-X. Claret, W. Wahli, and A. P. Wolffe.** 1993. A nucleosome-dependent static loop potentiates estrogen-regulated transcription from the Xenopus vitellogenin B1 promoter *in vitro*. EMBO J. **12:**423–433.
47. **Shachar, I., and R. A. Flavell.** 1996. Requirement for invariant chain in B cell maturation and function. Science **274:**106–108.
48. **Sinha, S., I.-S. Kim, K.-Y. Sohn, B. de Crombrugghe, and S. N. Maity.** 1996. Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. Mol. Cell. Biol. **16:**328–337.
49. **Sinha, S., S. N. Maity, J. Lu, and B. de Crombrugghe.** 1995. Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. Proc. Natl. Acad. Sci. USA **92:**1624–1628.
50. **Steimle, V., B. Durand, E. Barras, M. Zufferey, M. R. Hadam, B. Mach, and W. Reith.** 1995. A novel DNA-binding regulatory factor is mutated in primary MHC class II deficiency (bare lymphocyte syndrome). Genes Dev. **9:**1021–1032.
51. **Steimle, V., L. A. Otten, M. Zufferey, and B. Mach.** 1993. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell **75:**135–146.
52. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. Cell **83:**1091–1100.
53. **Ting, J. P.-Y., and A. S. Baldwin.** 1993. Regulation of MHC gene expression. Curr. Opin. Immunol. **5:**8–16.
54. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–8.
55. **Truss, M., J. Bartsch, A. Schelbert, R. J. G. Hache, and M. Beato.** 1995. Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter *in vivo*. EMBO J. **14:**1737–1751.
56. **Vilen, B. J., J. P. Cogswell, and J. P.-Y. Ting.** 1991. Stereospecific alignment of the X and Y elements is required for major histocompatibility complex class II DRA promoter function. Mol. Cell. Biol. **11:**2406–2415.
57. **Vilen, B. J., J. F. Penta, and J. P.-Y. Ting.** 1992. Structural constraints within a trimeric transcriptional regulatory region: constitutive and interferon-γ-inducible expression of the HLA-DRA gene. J. Biol. Chem. **267:**23728–23734.

57a. **Wright, K. L.** Submitted for publication.

58. **Wright, K. L., T. L. Moore, B. J. Vilen, A. M. Brown, and J. P.-Y. Ting.** 1995. Major histocompatibility complex class II-associated invariant chain gene expression is up-regulated by cooperative interactions of Sp1 and NF-Y. J. Biol. Chem. **270:**20978–20986.
59. **Wright, K. L., and J. P.-Y. Ting.** 1992. *In vivo* footprint analysis of the HLA-DR promoter: cell-specific interaction at the octamer site and up-regulation of X box binding protein by interferon-γ. Proc. Natl. Acad. Sci. USA **89:**7601–7605.
60. **Wright, K. L., B. J. Vilen, Y. Itoh-Lindstrom, T. L. Moore, G. Li, M. Criscitiello, P. Cogswell, J. Badley Clarke, and J. P.-Y. Ting.** 1994. CCAAT box binding protein NF-Y facilitates *in vivo* recruitment of upstream DNA binding transcription factors. EMBO J. **13:**4042–4053.
61. **Xie, X., T. Kokubo, S. L. Cohen, U. A. Mirza, A. Hoffmann, B. T. Chait, R. G. Roeder, Y. Nakatani, and S. K. Burley.** 1996. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. Nature **380:**316–322.
62. **Xing, Y., J. D. Fikes, and L. Guarente.** 1993. Mutations in yeast HAP2/HAP3 define a hybrid CCAAT box binding domain. EMBO J. **12:**4647–4655.
63. **Zeleznik-Le, N. J., J. C. Azizkhan, and J. P.-Y. Ting.** 1991. Affinity-purified CCAAT-box-binding protein (YEBP) functionally regulates expression of a human class II major histocompatibility complex gene and the herpes simplex virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **88:**1873–1877.